IN VITRO FUNGISTATIC ACTIVITY OF STILBAMIDINE, PROPAMIDINE, PENTAMIDINE AND DIETHYLSTILBESTROL*

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With the demonstration of their trypanocidal action by Lourie and Yorke in 1939 (1), a group of aromatic diamidines, notably stilbamidine (4,4′-stilbenedicarboxamidine), propamidine (p,p′-trimethylenedibenzamidine) and pentamidine (p,p′-pentamethylenedibenzamidine) has found a wide use in the treatment of trypanosomiasis. Subsequent studies revealed that these compounds possess as well activity against other protozoans as babesias and leishmanias, bacteria and fungi. Their development was recently reviewed by Schoenbach and Greenspan (2).

Propamidine was found by Fuller (3) and Thrower et al (4) to be active in vitro against gram positive cocci, although much less than against trypanosomes. It possessed *in vitro* bacteriostatic and chemotherapeutic activity in mice against *Clostridium welchii*, *Clostridium oedematiens* and *Clostridium septicemie* (5) in comparable order as the sulfonamides. Clinical trials using propamidine in the treatment of infected wounds and burns were successful, comparing favorably with results obtained from the use of sulfonamide drugs (4, 6, 7, 8). Elson (9) in 1945 re-investigated the antimicrobial action of propamidine and included a number of pathogenic fungi as test organisms. His findings revealed striking results in high dilutions against *Trichophyton sulfureum*, *Achorion schoenleinii*, *Sporotrichum schencki* and *Blastomyces dermatitidis*. Seabury and Artis (10) in their study of the susceptibility of *Histoplasma capsulatum* to therapeutic agents found that this organism was sensitive to stilbamidine in a dilution of 10 mg. %. More recently, Snapper et al (11) confirmed the sensitivity of *Blastomyces dermatitidis* and *Histoplasma capsulatum*, both in their mycelial and yeast phases, to dilutions of a few micrograms of stilbamidine or of 2-hydroxystilbamidine per ml. of medium. The 2-hydroxystilbamidine was recommended by the authors on account of the absence of toxic neuropathy, especially of the trigeminal nerve, as encountered with stilbamidine administration. Still further confirmatory evidences were reported as to the fungistatic action of stilbamidine against *Blastomyces dermatitidis* (12).

These *in vitro* studies were followed by *in vivo* experiments and clinical trials

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Read by title before the 25th Annual Meeting of the Central Society for Clinical Research held in Chicago, Illinois on November 7–8, 1952.

Stilbamidine, propamidine and pentamidine were supplied by Merck & Co. and Merrell & Co.

Received for publication April 13, 1953.

on cases of mycoses. The only study so far reported using experimental animals is that of Heilman (13) wherein he obtained definite suppression by stilbamidine of pulmonary infection of mice with *Blastomyces dermatitidis* given intravenously. Infections of the brain among these mice, however, were not controlled. Seabury (14) in his two cases of histoplasmosis treated with stilbamidine reported at most only initial and temporary improvement with no significant effect on the course of the disease. No favorable response was also obtained in three cases of cryptococcosis given stilbamidine (15). It is in the treatment of both cutaneous and systemic North American blastomycosis that encouraging results were encountered (11, 12, 15, 16, 17, 18). A patient with actinomycosis resistant to penicillin and sulfadiazine responded promptly to the administration of stilbamidine (19).

Diethylstilbestrol (4,4′-dihydroxy-a,a′-diethylstilbene) followed a different course of development. In 1938, Dodds et al (20) during the course of their study of estrogenic compounds became impressed with the extreme potency of diethylstilbestrol and it has found a wide clinical application as an estrogen since then. Beside this better known estrogenic property, diethylstilbestrol has in addition remarkable *in vitro* antibacterial activity against gram positive organisms in the order of 1:50,000 to 1:500,000 as the minimum effective concentration (21, 22, 23). Faulkner (24) reported bactericidal activity against *Mycobacterium tuberculosis* in a range of 1:5,000 to 1:100,000 minimum lethal concentration. A single dose of 40–50 rat units of stilbestrol was found by Foley and Aycock (25) to render mice highly resistant to a dose of mouse virulent hemolytic streptococci which regularly killed normal mice of the same sex and age. The estrogen was given 48 hours before the mice were infected with the streptococci and the authors suggested that the protection was due to a stilbestrol-induced factor which interfered with the spread of the organisms.

Investigations on the *in vitro* antifungal activity of diethylstilbestrol are relatively meager. Harder (26) in studying the effects of diethylstilbestrol on superficial mycotic infections supplemented his clinical findings with *in vitro* experiments wherein the lowest inhibiting concentrations were determined as 1:20,000 for *Microsporum gypseum* and *Trichophyton schoenleinii*; 1:40,000 for *Microsporum audouini*, *Microsporum canis* and *Trichophyton mentagrophytes*; 1:60,000 for *Trichophyton rubum* and *Epidermophyton floccosum*; and 1:100,000 for *Blastomyces dermatitidis* and *Histoplasma capsulatum*. Reiss (27) included diethylstilbestrol in a group of sex hormones he was testing for possible fungicidal effects and found it effective in retarding the growth of *Trichophyton rubrum*, but not of *Trichophyton gypseum*. He expanded this study using diethylstilbestrol and 11 steroid hormones with 14 pathogenic fungi as test organisms and still found diethylstilbestrol most effective, particularly against *Microsporum audouini*, *Epidermophyton inguinale*, *Histoplasma capsulatum* and *Actinomyces transylvanensis* (28). In another investigation, diethylstilbestrol was reported by Heinemann (29) to possess fungistatic activity at levels of 1:40,000 to 1:80,000 dilution against *Trichophyton purpureum*, *Trichophyton sulfureum*, *Epidermophyton floccosum*, *Microsporum audouini*, *Achorion schoenleinii* and *Coccidioides immitis*.

Based on the observation that tinea capitis exhibited spontaneous involution at puberty, diethylstilbestrol was tried on account of its hormonal effects in the treatment of tinea capitis locally and/or orally with variable results. Favorable responses were obtained by Poth and Kaliski (30), by Law (31) and by Harder (26), but the species of the causative organisms were not determined for the individual cases in these studies. Failures were reported by Lewis, Hopper and Reiss (32), by Felsher (33) and by Dobes (34). *Microsporum audouini* was isolated from most of the cases of tinea capitis used in the latter investigations.

As regards the use of diethylstilbestrol in deep mycosis, Curtis and Harrell (12), noting the similarity of the chemical structures of diethylstilbestrol and stilbamidine, reported the first two cases of cutaneous North American blastomycosis with regression under diethylstilbestrol therapy.

Other hormones were found to have certain action on pathogenic fungi. The first studies along these lines were reported by Hruszek (35) who obtained definite inhibition of growth of *Microsporum audouini*, *Trichophyton gypseum* and *Trichophyton schoenleinii* by prolan extracted from the anterior pituitary gland and, to a lesser extent, by folliculin. Of the hormones tested by Reiss (27) methyltestosterone had a more definite fungistatic effect on the growth of *Trichophyton purpureum* and *Trichophyton gypseum* than alpha-estradiol and desoxycorticosterone. When topically applied, it was also more effective than alpha-estradiol against experimental *Trichophyton purpureum* infection in castrated rabbits (36). Both were, however, ineffectual when given by injections. More recently, Nekam and Polgar (37) confirmed the fungistatic activity of sex hormones containing the sterol radical as well as of the stilbene derivatives.

Methyltestosterone was included in the present study because of the above findings and because of the more favorable results obtained with it during preliminary screening fungistatic tests of different steroid hormones done in this laboratory. Isonicotinic acid hydrazide was also tested because of the present interest on its bacteriostatic activity against *Mycobacterium tuberculosis* and the observation that it may possess some slight antifungal properties (38).

In view of the results of these scattered studies on the stilbene derivatives, it seemed worthwhile to investigate further in detail the comparative fungistatic spectra of these compounds.

METHODS

Stock solutions of known concentrations of each compound tested were prepared first in appropriate solvents. Distilled water was used for dissolving stilbamidine, propamidine, pentamidine and isonicotinic acid hydrazide (cotinazin, Pfizer) and acetone for diethylstilbestrol and methyltestosterone. Calculated amounts of the stock solutions were incorporated into Sabouraud's dextrose agar and blood agar that had been autoclaved and cooled to about 50°C. The addition was made with sufficient shaking to insure uniformity in solution. The resulting final dilutions of each compound in the agar medium were in a series of 1 mg./ml. medium, 0.1 mg./ml., 0.01 mg./ml. and 0.001 mg./ml. It was found necessary to extend these dilutions in some instances to 0.1 microgram/ml. medium, 0.01 microgram/ml. and 0.001 microgram/ml. These were set up in tube slants.

Stilbamidine, propamidine and pentamidine are used in this report in terms of their isethionate salts.

Young vigorous colonies of each test organism were obtained and as uniform suspensions

as possible of the organisms in physiological salt solution were made. Uniformity of suspension was easily achieved with the yeast forms, but with certain difficulties among the filamentous colonies. One tenth ml. of each of these suspensions were inoculated into the prepared slants.

The final set-up was made such that each test organism which numbered 26 was tested against the serial dilutions of each of the six compounds used in the investigation. All the experiments were done in duplicates and properly controlled. Controls for the compounds dissolved in acetone were set up by adding 1.0% of acetone to Sabouraud's dextrose agar slants which was the amount used in making up the serial dilutions.

The cultures were left at room temperature and observed closely every week up to six weeks, even up to twelve weeks for very slowly growing cultures. In the cases of the dimorphic species of *Blastomyces dermatitidis*, *Blastomyces braziliensis* and *Histoplasma capsulatum*, similar experiments were performed using blood agar dextrose slants incubated at 37°C.

Results that revealed promising inhibitions were checked with repeat experiments.

Experiments using infected clinical materials were also performed. Instead of suspensions of cultures of the organisms, positive KOH skin scrapings and fluorescing hairs to Wood's light were used as inoculants on slants of serial dilutions of diethylstilbestrol as set up in the previous experiments in triplicates. These were similarly controlled and observed. The skin scrapings were taken from a case of tinea corporis which revealed growth of Trichophyton mentagrophytes. The hairs were obtained from three cases of tinea capitis caused by *Microsporum audouini*.

RESULTS AND DISCUSSION

In view of the possible influence of changes in pH of the medium brought about by the addition of the compounds, the pH of representative samples of each medium was determined. The greatest change in pH was found to be pH 0.2. This was considered not big enough of a change to significantly affect the growth of the fungi tested inasmuch as these different species are proven to possess the ability to grow in a remarkably wide range of pH.

Results obtained are presented in Table I. It is to be noted that of the compounds tested, diethylstilbestrol had the widest range and greatest activity against the species used as test organisms. It was effective at dilutions of 1 microgram/ml. medium against *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Blastomyces braziliensis* and *Trichophyton ferrugineum* and at a level of 10 microgram/ml. against *Trichophyton rubrum*, *Trichophyton schoenleinii*, *Trichopyton violaceum*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, *Nocardia asteroides* and *Coccidioides immitis*. Its *in vitro* efficacy against *Coccidioides immitis* seems to warrant further investigations.

Most of the strains were inhibited by stilbamidine, propamidine, and pentamidine at a concentration of 1 mg./ml. medium. Stilbamidine showed activity in lower concentrations only against *Hormodendrum pedrosoi* and *Blastomyces dermatitidis* and pentamidine only against *Hormodendrum pedrosoi* and *Sporotrichum schencki*. Propamidine had a wider spectrum, being effective at higher dilutions against *Trichophyton ferrugineum*, *Cladosporium wernecki*, *Hormodendrum pedrosoi*, *Phialophora verrucosa*, *Sporotrichum schencki*, *Blastomyces dermatitidis*, *Blastomyces braziliensis*, and *Coccidioides immitis*.

Two strains of *Blastomyces dermatitidis* were examined. One was freshly isolated from a case of pulmonary blastomycosis with dissemination to the subcutaneous tissue and skin. The other presented an opportunity for sensitivity

TABLE I

The lowest effective concentrations in mg. of compound tested per ml. medium against fungi

	STILBAMIDINE	PROPAMIDINE	PENTAMIDINE	DIETHYLSTILBESTROL	METHYLTESTOSTERONE	ISONICOTINIC ACID
Trichophyton mentagrophytes	1.0	>1.0	>1.0	0.1	>1.0	>1.0
Trichophyton rubrum	1.0	1.0	1.0	0.01	0.1	>1.0
Trichophyton schoenleini	1.0	1.0	1.0	0.01	>1.0	>1.0
Trichophyton violaceum	1.0	1.0	1.0	0.01	0.1	>1.0
Trichophyton ferrugineum	1.0	0.1	1.0	0.001	0.1	>1.0
Trichophyton tonsurans	1.0	1.0	1.0	0.01	0.1	>1.0
Microsporum gypseum	1.0	1.0	1.0	0.1	>1.0	>1.0
Microsporum audouini	1.0	1.0	1.0	0.1	>1.0	>1.0
Microsporum canis	1.0	1.0	1.0	0.1	>1.0	>1.0
Epidermophyton floccosum	1.0	1.0	1.0	0.01	0.1	>1.0
Cladosporium wernecki	1.0	0.1	1.0	0.1	>1.0	>1.0
Hormodendrum pedrosoi	0.01	0.1	0.1	>1.0	>1.0	>1.0
Phialophora verrucosa	1.0	0.1	1.0	>1.0	>1.0	>1.0
Monosporium apiospermum	1.0	1.0	1.0	0.1	>1.0	>1.0
Nocardia asteroides	1.0	1.0	1.0	0.01	>1.0	>1.0
Candida albicans	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
Cryptococcus neoformans	1.0	>1.0	>1.0	>1.0	>1.0	>1.0
Sporotrichum schencki	1.0	0.01	0.001	1.0	>1.0	>1.0
Blastomyces dermatitidis* (Heaney strain) Room temp.	0.1	0.1	1.0	0.001	>1.0	>1.0
Blastomyces dermatitidis* (Heaney strain) 37°C	0.01	0.01	1.0	0.01	>1.0	>1.0
Blastomyces dermatitidis† (Gordon strain) Room temp.	0.1					
Blastomyces dermatitidis† (Gordon strain) 37°C	0.1					
Blastomyces dermatitidis‡ (Gordon strain) Room temp.	1.0	0.1	1.0	0.01	1.0	>1.0
Blastomyces braziliensis Room temp	1.0	0.01	1.0	0.001	0.1	>1.0
Blastomyces braziliensis 37°C	1.0	0.1	0.1	0.01	0.1	>1.0
Histoplasma capsulatum Room temp	1.0	1.0	1.0	0.001	0.1	>1.0
Histoplasma capsulatum 37°C	0.1	0.01	1.0	0.1	0.1	>1.0
Coccidioides immitis	1.0	0.1	1.0	0.01	>1.0	>1.0
Alternaria sp	0.1	0.01	1.0	>1.0	>1.0	>1.0
Penicillium sp	1.0	>1.0	>1.0	>1.0	>1.0	>1.0
Aspergillus niger	1.0	1.0	>1.0	>1.0	>1.0	>1.0

* This strain was freshly isolated from the sputum of an active untreated case of systemic blastomycosis.

† This original isolate was obtained from a case of cutaneous and systemic blastomycosis before stilbamidine therapy.

‡ This was isolated from a recurrent subcutaneous abscess from the same patient after having had two courses of stilbamidine with remissions.

tests before and after administration of stilbamidine. This was originally isolated from a patient who had systemic and cutaneous blastomycosis. The second isolate was obtained ten months later, after the patient had had essentially two courses of stilbamidine with remissions and recurrences. The first course was

divided into 100 mg. of stilbamidine given intravenously daily for 6 days in August, 1951; 150 mg. for 12 days in September, 1951; and 150 mg. for 10 days in October, 1951. With these, the patient improved remarkably and was discharged. However, he returned two months later with recurrent lesions and was given the second course of 150 mg. stilbamidine daily for 22 days in January, 1952. Again, he responded favorably but only to return three months later with another recurrence at which time the second isolate of this strain was taken. This second isolate was completely inhibited by stilbamidine only at a concentration of 1 mg./ml. medium whereas the original isolate taken before any stilbamidine therapy was inhibited at a concentration of 0.1 mg./ml. This, plus the clinical recurrences, may be suggestive of the acquisition of resistance to stilbamidine by the organism.

Methyltestosterone revealed a certain amount of fungistasis against some dermatophytes, *Blastomyces dermatitidis*, *Blastomyces braziliensis* and *Histoplasma capsulatum*.

Isonicotinic acid hydrazide showed no fungistatic activity at the range of concentrations used.

The nonpathogenic species—alternaria, penicillium and aspergillus—were resistant, except for some sensitivity of alternaria to stilbamidine and propamidine. Among the pathogenic species studied, *Candida albicans*, *Cryptococcus neoformans* and *Nocardia asteroides* seem to be more resistant.

The lowest concentration of diethylstilbestrol where no growth appeared after implantation with skin scrapings from a KOH positive case of tinea corporis infection by *Trichophyton mentagrophytes* was 0.1 mg./ml. medium. The fluorescent hairs from three cases of tinea capitis caused by *Microsporum audouini* did not reveal growth at the lowest concentrations of 0.1 mg./ml. in one case and 0.01 mg./ml. in two cases. These inhibiting concentrations are more or less in the same order as those obtained using subcultures of the organisms. All controls in these experiments were positive.

It seems worthwhile to extend these comparative screening tests for fungistatic activity to other stilbene derivatives and related compounds as had been notably done regarding their bacteriostatic and bactericidal properties. It is unfortunate that these investigations were practically cut short with the eminently successful use of the sulfonamide derivatives and the antibiotics in bacterial infections, but results already suggested that quite a number of these stilbenes were active against bacteria *in vitro*. Brownlee et al (21) and Faulkner (23) found 4-hydroxy-a,b-diethylstilbene to be the most active compound in their series which also included diethylstilbestrol. Similarly, fungistatic activity may also not be limited to the four related compounds presently studied. The search for other stilbenes that may show equal, if not greater, fungistatic activity than that shown by diethylstilbestrol is more desirable in view of the probability that the potent estrogenic property of diethylstilbestrol may become a definite limitation in its use clinically as a chemotherapeutic agent. Such a compound possessing high fungistatic without estrogenic activity may be existent. Curtis and Harrell (12) suggested that the clinical effects of the stilbenes on mycosis were chemotherapeutic and not simply hormonal. The degree of bactericidal properties of these

compounds has been shown to be uncorrelated with their estrogenic abilities (21, 22, 23, 39, 40).

SUMMARY

1. Using the Sabouraud's agar dilution method, the fungistatic spectra of stilbamidine, propamidine, pentamidine, diethylstilbestrol, methyltestosterone and isonicotinic acid hydrazide were determined against 22 species of pathogenic and 3 species of nonpathogenic fungi.

2. The order of these compounds as regards their *in vitro* fungistatic activity was as follows: diethylstilbestrol, propamidine, stilbamidine, pentamidine and methyltestosterone. Isonicotinic acid hydrazide showed no demonstrable fungistasis.

3. The sensitive strains of pathogenic fungi included a number causing systemic mycoses, as *Blastomyces dermatitidis*, *Blastomyces braziliensis*, *Histoplasma capsulatum*, *Sporotrichum schencki* and *Coccidioides immitis*.

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